

IDENTIFICATION, MASS PRODUCTION AND APPLICATION OF PIGMENT IN FOOD INDUSTRY ISOLATED FROM *MONASCUS SP*

ANCHANA DEVI C & MEERA J

PG & Research, Department of Biotechnology, Women's Christian College, Chennai, Tamil Nadu, India

ABSTRACT

The fungi *Monascus* was isolated from pomegranate. The morphological characteristics of *Monascus* were identified using Sabouraud Dextrose Agar (SDA) and Sabouraud Dextrose Broth. Microscopic view showed the presence of conidia. Soxhlet extraction was done to obtain the pigment from the sample. After several hours of extraction, the pure pigment was collected. The red pigment was observed after many cycles of extraction, the solvent was evaporated and the pigment was collected separately. UV spectroscopy was adjusted to 450 nm, the value obtained in the UV Spectroscopy for sample 1 was 0.654 and for the sample 2 was 0.666 where, the value of sample 2 was higher than sample 1. The maximum growth of the fungi and pigment production was seen in flask 2 and flask 3 where the pH maintained at 6, room temperature at 37°C. The additional amount of rice bran which was used as a carbon source assisted in the high rate of pigment production in SSF. The pigment Monascorubin and Monascin was found to be present in the sample. The R_f values obtained were 0.72 corresponds to orange pigment, 0.818 indicates the presence of yellow pigment and the value 0.25 showed the presence of red coloured compound. On application of the isolated pigment to raw cheese as preservative, it was observed even after 5 days of addition, there was no rotting of the cheese. Hence the pigment acted as a good source of natural preservative.

KEYWORDS: Pomegranate Fruit, *Monascus*, Solid State Fermentation, Soxhlet Extraction and Thin Layer Chromatography

INTRODUCTION

There are about 500 organisms of fungi which are used for the fermentation of food products. (Yeun Kim et al., 2010) Processing the food with fungi makes it consumable by adding, modifying, including flavours, nutritional elements such as vitamins or colour to enhance the appearance. *Monascus* species was obtained by culturing seeds and fruits of Pomegranate in media. This species belong to the family Monascaceae of the phylum Ascomycota. There are 9 species which belong to the *Monascus* family. These are *M. pilosus*, *M. ruber*, *M. purpureus*, *M. floricornis*, *M. eremophilus*, *M. pallens*, *M. sanguineus*, *M. lunisporus*, and *M. argentinensis*. The morphological characteristic feature of *Monascus* is a fluffy white colored colony on the SDA plate. The characteristics of the fungi *Monascus* is white cotton like appearance on the SDA plate. *Monascus* pigments are polyketides and oxygen is an essential substrate for their biosynthesis. (Carvalho J et al., 2003). It is observed that the final high viscosity of the culture broth in the late log phase interfered with the aeration of the cultures. Their therapeutic properties and their relatively high stability with respect to pH and temperature where the interesting features that promote their use as substitutes for synthetic colorants. Antibacterial activity of the purified pigment was tested and found active only against Gram positive bacteria. *Monascus* sp can produce many pigments. (Rashmi D and Padmavathi T, 2013) Yellow pigments: Monascin ($C_{21}H_{26}O_5$) and Ankaflavin ($C_{23}H_{30}O_5$);

Orange pigments: Monascorubrin ($C_{23}H_{26}O_5$) and Rubropunctatin ($C_{21}H_{22}O_5$); and Red pigments: Monascorubramine ($C_{23}H_{27}NO_4$) and Rubropuntamine ($C_{21}H_{22}O_4$). Monascus sp., which is known to produce various high value secondary metabolites such as lovastatin, γ -amino butyric acids (GABA), Lovastatin is one of the most studied secondary metabolites of Aspergillus and is considered to be a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is a rate limiting and regulatory enzyme of cholesterol biosynthesis. The potential of the natural substrate for the biopigment production is studied in this paper and solvent used for extracting the pigment was also studied.

MATERIALS AND METHODS

Isolation of Fungi

The medium was prepared using Sabouraud Dextrose Agar (SDA). 16.25g of SDA g/L (Dextrose: 40.000, Mycological, peptone 10.000, Agar 15.000, Final pH (at 25°C) 5.6 ± 0.2) were taken in a conical flask with 250mL distilled water according to (Babitha S *et al.*, 2006). The flask was kept for autoclave for 20 minutes. The medium was poured into the petriplate and allowed for the agar to solidify. The pomegranate fruits and seeds were inoculated in the petriplate. The petriplate was kept in dark condition for 25 days.

Solid State Fermentation

Required amount of the fungal mass was isolated separately during filtration. The medium was prepared in 250ml flask. The flask containing the broth medium was autoclaved for 20 min. 5g of rice bran was weighed and used as a substrate (Julio C Carvalho *et al.*, 2007). Substrate was mixed in the flask and kept in shaker for 10min. Four samples were prepared and maintained at different temperature and pH. pH for the samples were adjusted using the pH meter by adding NaOH and HCL (pH. 2, 4, 6, 10), (Azza S. Naik & Smita S. Lele, 2012). Then the samples were maintained at temperatures (50°C, 37°C, 14°C). (Bum-Kyu Lee *et al.*, 2002)

Dry Cell Weight

The mycelia obtained from the solid state fermentation were filtered using Whatmann filter paper. The solid mycelium was weighed using petriplate. The empty preweight of the plate was noted and then with the sample the weight was determined.

Soxhlet Extraction

The Soxhlet extractor helps in extracting the pigment from the sample by using a suitable solvent. The sample was sealed using the filter paper and solvent used was ethanol: distilled water (95:5). The solvent in the extractor along with the sample is heated continuously with the help of a heating mantle at the bottom. The solvent vapour travels to the distillation arm and returns to the chamber. The condenser at the top helps in cooling the solvent vapour. (Dan Chen and Zanmin Wu, 2005) The desired compound in the sample dissolves within the solvent. After many cycles of extraction, the compound settles in the distillation flask and it is collected separately. The compound in the flask was further heated for the remaining solvent to be evaporated. Thus the pure compound was obtained. (Ju *et al.*, 1994)

Thin Layer Chromatography

The solvent chloroform was used as a mobile phase for the compound in the sample to be identified. Silica slurry coated TLC plate was used as a stationary phase. A line was drawn at the bottom of the TLC plate, the sample was placed using the capillary tube over the marked line. The TLC plate was placed in a beaker containing the mobile

phasechloroform(100%) and was left undisturbed for the solvent to reach the top of the TLC plate (Hin –Chung Wong and Philip E. Koehler, 2006).The TLC plate was removed and air dried. The pigment was identified by observing it under UV transilluminator.

The Retention factor (R_f) of the compound was calculated using the formula

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Distance travelled by the solvent front

Antimicrobial Activity

The Mullerhilltonagar was prepared, autoclaved and poured in petriplate and allowed for the agar to solidify. The culture organisms were inoculated using sterile swab. Well was punctured on the agar. The control and samples were loaded in the well (Leonard G et al., 2013). The plates were incubated for 24 hours. Zones of inhibition were observed and the results were tabulated.

RESULTS AND DISCUSSIONS

The fungi growth was observed on the 25th day of inoculation in SDA plate. The isolated fungi were identified by the white cotton colony appearance on the fruit pulp.



Figure 1: Pigment Production by *Monascus* sp at 25th Day

The orange coloured pigment was obtained on 27th day of incubation.



Figure 2: Pigment Production in SD Broth

The sample kept for mass cultivation showed the production of pigments which was stable over the wide range of pH. The coloured pigment was observed without the addition of carbon or nitrogen source. (Tseng YY et al., 2000)

SOLID STATE FERMENTATION

Solid state fermentation was done using rice bran as a substrate the samples were kept at different temperature (14°C, 37°C and 50°C) and the pH was maintained at 2, 4, 6 and 10.

Table 1: Temperature and pH for Solid State Fermentation (SSF)

Flask	pH	Temperature
FLASK I	2	Hot air oven (50°C)
FLASK II	4	Room temperature(37°C) with shaker
FLASK III	6	Room temperature(37°C)
FLASK IV	10	Freezer (14°C)

The maximum growth of the fungi and pigment production was seen in flask 2 and flask 3 where the pH maintained was 6 and incubated in room temperature at 37°C. The additional amount of rice bran which was used as a carbon source assisted in the high rate of pigment production in SSF. Effect of glycerol as a carbon source and malt as nitrogen source was used in two strains of *M. sanguineus* and *M. purpureus*.

SOXHLET EXTRACTION

Soxhlet extraction was done to obtain the pigment from the sample. After several hours of extraction the pure pigment was collected. (Tallapragada, P et al., 2009)



Figure 3: Soxhlet Extractor



Figure 4: Extraction after 3 Hours

The red pigment was observed after many cycles of extraction the solvent was evaporated and the pigment was collected separately. The pigment collected was tested for its antimicrobial activity and the results were tabulated. (Table 2)

Table 2: Antimicrobial Activity of the Pigment Obtained from Soxhlet Extraction

Species	Volume of Extract (Fraction)	Diameter of Zone of Inhibition (mm)
<i>S.aureus</i>	50 μ l	00
<i>Pseudomonas sp</i>	50 μ l	1mm
<i>Streptococcus Sp</i>	50 μ l	00
<i>Enterococcus sp</i>	50 μ l	00
<i>Enterobactersp</i>	50 μ l	00
<i>Bacillus sp</i>	50 μ l	22mm
<i>E.colisp</i>	50 μ l	0.5mm

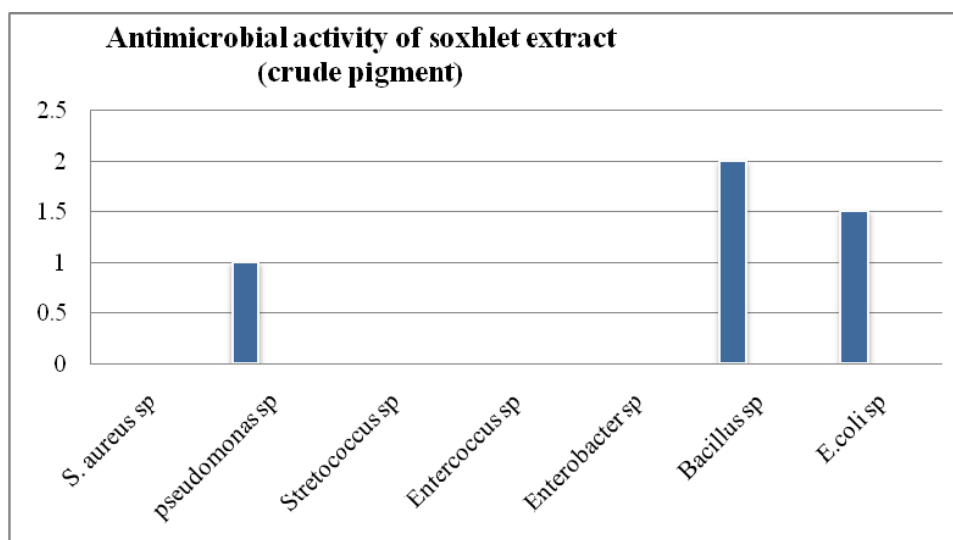


Figure 5: Graph Representing the Antimicrobial Activity of the Pigment

Thin Layer Chromatography

TLC was done using chloroform (100%) as a mobile phase for the identification of the compound. When the TLC

plate was removed and air dried the pigment was identified by observing under the UV transilluminator



Figure 6: Pigment on TLC Plate



Figure 7: TLC Plate under UV Transilluminator

The Retention factor (R_f) of the compound was calculated using the formula

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Distance travelled by the solvent front

Retention factor values of the sample from the Soxhlet crude pigment:

Table 3: R_f Values obtained in Thin Layer Chromatography

Sample 1	Sample 2
0.72	0.818
0.8	0.72
0.333	0.25

The pigment production was determined using the R_f values where in the sample orange, yellow and red pigments were obtained. The R_f values obtained 0.72 indicated the presence of orange pigment, 0.818 indicated the presence of yellow pigment and the value 0.25 showed the presence of red coloured compound. The obtained values from TLC correlated with the values reported by Babitha S et al., were similar. Thus the pigment was identified as Monascorubin and Monascin found to be present in the sample.

APPLICATION

The red coloured pigment Monascin was extracted by Soxhlet method. The coloured pigment was added to rice porridge as a colouring agent. The preservative property was observed by addition of the pigment to homemade cheese. The sample was incubated in dark condition. After 5 days of observation, the cheese retained its original texture. Hence it was proved to be a natural colouring and preservative agent.



Figure 8: Coloured Pigment Tested in Porridge



Figure 9: Pigment Addition in Homemade Cheese



Figure 10: Preservative Property observed after 5 Days of Incubation

1: Homemade cheese with pigment 2: Homemade cheese without pigment

The preservative property was observed by taking 2g of cheese in crucible and the 10 μ l of pigment was added to the crucible 1. Two crucibles were incubated in room temperature in dark condition. Whey was observed in crucible 2 after 24 hours of incubation. On the second day the texture of the cheese in crucible 2 changed. After 5 days of serial observation the cheese in crucible 1 did not form whey and no change in texture was observed. Hence the pigment proved to be an excellent natural preservative.

CONCLUSIONS

From the results obtained the pomegranate fruit is an effective source for the production of pigments from *Monascus* sp. The fungi were characterized microscopically by the presence of hyphae and spores using LPCB stain. The

fungal layer and the pigment production were observed in mass cultivation which was incubated for 20 days. Solid state fermentation was done in different temperature and different pH and maximum growth was obtained in pH 6 in 37°C. The pigment was filtered using Whatmann filter paper and extracted using Soxhlet extraction. The pigment was collected. Antimicrobial activity of the pigment was seen and showed activity against *Pseudomonas* sp, *Bacillus* sp and *E. coli* sp. UV spectroscopic analysis was done and the values were obtained ranging from 0.654 to 0.666. TLC was done to identify the compound and the pigment in the sample were Monascin and Monascorubin. The colouring property and preservative property was observed in porridge and homemade cheese. The pigment thus proved to be natural source of colourant and preservative of cheese.

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